Reference: *Biol. Bull.* **186:** 328–341. (June, 1994)

Biological Characteristics and Biomedical Applications of the Squid *Sepioteuthis lessoniana* Cultured Through Multiple Generations

PHILLIP G. LEE, PHILIP E. TURK, WON TACK YANG, AND ROGER T. HANLON

Marine Biomedical Institute, University of Texas Medical Branch, Galveston, Texas 77555-0863

Abstract. Providing squids—especially their giant axons—for biomedical research has now been achieved in 10 mariculture trials extending through multiple generations. The noteworthy biological characteristics of *Sepioteuthis lessoniana* are (1) this species is behaviorally and morphologically well suited to the laboratory environment; (2) the life cycle is completed in 4–6 months; (3) growth is rapid (12% and 5% wet body weight d^{-1} for 100 d and for the life span, respectively), with adult size ranging from 0.4–2.2 kg; (4) feeding rates are high (30% wet body weight d^{-1}), and a variety of live crustaceans and fishes are eaten; (5) crowding is tolerated (about 4 squids m^{-3}); (6) the incidence of disease and cannibalism is low; and (7) reproduction in captivity allows culture through three successive generations. Engineering factors contributed to culture success: (1) physical design (*i.e.*, size, shape, and painted pattern) of the culture tanks; (2) patterns of water flow in the culture tanks; (3) water filtration systems; and (4) spawning substrates. Initial production (a few hundred squids per year) suggests that large-scale culture will be able to supply the needs of the biomedical research community. The size (>400 μm in diameter) and characteristics of the giant axons of *Sepioteuthis* are appropriate for experimentation, and other studies indicate that the eye, oculomotor/equilibrium system, olfactory system, blood, and ink are equally suitable for research.

Introduction

The squid giant axon is a valuable preparation in biomedical research. Squids have been used as research models, not only for neuroscience, but for physiology (cardiac, circulatory, sensory and muscle), immunology, molecular biochemistry, nutritional biochemistry, oncology, aging, and ethology (Gilbert *et al.*, 1990). Squids also have commercial importance since they are eaten regularly in many regions of the world, especially the Orient and Southern Europe (Asian Development Bank/Infofish, 1991). To date no commercial culture projects have been initiated, but limited stocking programs have been investigated in Japan (Sato and Tsuzaki, 1984; Japan Sea Farming Fisheries Association, 1985; Nagata and Hirata, 1986).

Attempts to culture squids during the last 50 years have been unsuccessful due to the organism's small hatching size, unknown dietary habits, active behavior, and susceptibility to skin damage and disease resulting from captivity (Hanlon, 1990; Hanlon *et al.*, 1991). Only the loliginid species *Loligo opalescens* has ever been cultured from field-collected eggs to the first laboratory-spawned generation, demonstrating the potential for mass culture of squids (Yang *et al.*, 1983, 1986).

Compared to other loliginid squid species, *Sepioteuthis lessoniana* Lesson, 1830 (Figs. 1 and 2) appears to be the most adaptable to the laboratory environment (Hanlon *et al.*, 1991). It is a neritic species distributed throughout the Indo-West Pacific (Okutani, 1980; Dotsu *et al.*, 1981; Lu and Tait, 1983), and it is valuable to the Japanese fishery (Suzuki *et al.*, 1983). Laboratory studies of *S. lessoniana*, conducted by Choe and Oshima (1961), Choe (1966a,b), Inoha and Sezoko (1968), Saso (1979), Tsuchiya (1982) and Segawa (1987), have described the early life stages and life cycle and have verified growth rates estimated from fishery data (reviewed by Segawa, 1987). Several of these culture trials spanned much of the life cycle (200–300 d) and demonstrated high growth rates (260 mm mantle length, ML, in 306 d; Tsuchiya, 1982).

Received 19 May 1993; accepted 1 March 1994.

Abbreviations: ML, dorsal mantle length; BW, wet body weight; IGR, instantaneous growth rate; G_p, parental generation; G_1, first laboratory generation; G_2, second laboratory generation; G_3, third laboratory generation; scuba, self-contained underwater breathing apparatus.

Figure 1. Adult *Sepioteuthis lessoniana* 280 mm ML and 2.21 kg BW after 194 days of culture. Note the extensive fin that is characteristic of the genus.

LaRoe (1971) reared the smaller Caribbean species *S. sepioidea* to 77 g in 146 d. The consistency of successful egg incubation and hatchling culture and the large size attained in the laboratory suggested that *S. lessoniana* might possess developmental, physiological, and behavioral characteristics suited to laboratory culture.

We have cultured this species continuously since September 1987 and have made 10 culture trials. *S. lessoniana* is unique among the larger loliginid species for its tolerance to handling and confinement, and it thus has great potential both as a non-mammalian model for biomedical research and as a commercial mariculture species. Our principal objectives were to evaluate and quantify the suitability of the species for culture in terms of growth, behavior, reproduction, and environmental tolerances. This report describes in detail 2 of the 10 culture trials, as they represent the major features of this species in captivity. In addition, *S. lessoniana* was evaluated concurrently for use as a giant axon preparation and for other biomedical research studies. Trial One was the first successful attempt to culture squids through multiple generations: the parental generation (G_p) originated from eggs collected in the field and was followed by first (G_1), second (G_2), and third (G_3) laboratory generations spawned in laboratory culture tanks. Trial Two was a subsequent production experiment in which larger numbers of parental generation (G_p) squids were cultured from field-collected eggs.

Materials and Methods

Egg transport

Sepioteuthis lessoniana egg strands for Trial One (G_p) were collected by a scuba diver (R. T. Hanlon) from rock and seagrass substrates and, prior to shipping, were held in flow-through aquaria at Kominato Laboratory, Tokyo University of Fisheries, Chiba Prefecture, Japan. The egg strands were shipped in 2-mil-thick plastic bags secured in polystyrene boxes, measuring 71 cm L × 39 cm W × 26 cm H, with 3.5-cm-thick walls. Fresh filtered seawater and gaseous O_2 were added to the bags in equal volumes (7 l total volume). Frozen chemical ice packs wrapped in newspaper were added to each box for a ratio of 150 g ice l^{-1} of seawater. The number of egg strands per bag, the volume of shipping water per egg strand, and the developmental stages (Segawa, 1987) of the embryos were varied among the bags so that changes in water quality (pH and NH_4-N) could be evaluated through a range of packing densities. When the eggs arrived in Texas, they were acclimated to the temperature, salinity, and water conditions of the hatchery tanks; *i.e.*, culture water was added to the shipping bags at a rate that limited temperature change to 0.5°C h^{-1}. Eggs for Trial Two (G_p) were collected, with the aid of scuba, from submerged trees in Tokushima Prefecture, Japan; the eggs were handled similarly to Trial One.

Laboratory-spawned eggs (G_1, G_2, and G_3) were removed from the production tanks and placed in hatchery tanks. Artificial substrates mimicking sea grass (plastic aquarium grass) or reef structures (carbonate rock and polyvinylchloride pipe) were provided for spawning. Frequently, artificial egg strands (silicone strands; Yang *et al.*, 1986) were tied to these substrates to further induce egg laying. The temperature and salinity of the production and hatchery tanks were matched prior to transfer, and the eggs were kept submerged at all times.

Tank characteristics, egg care, and hatching

Squid eggs, whether laboratory-spawned or field-collected, were incubated in 1.8-m circular hatchery tanks (described in Yang *et al.*, 1989; Turk and Lee, 1991) con-

Figure 2. Young *Sepioteuthis lessoniana* (*ca.* 60 days) showing a response to disturbance that can be shown even by hatchlings. Note the elaborate body patterning and posture.

taining natural seawater collected in the Gulf of Mexico at least 80 km offshore from Galveston, Texas. These tanks were equipped with a screened central core where the water was removed by siphon to the filter tank. The 1.8-m circular filter bed included an undergravel biofilter and protein skimmer. Filtered water was drawn from beneath the biofilter and pumped back to the culture tank through a particle filter (35-μm pore diameter), an activated carbon filter, and two 30-W ultraviolet (UV) sterilizers; the filtered water was discharged at the water surface through 2.5-cm spray bars. The flow pattern was circular (2.5 cm s^{-1}, measured halfway between the core and the tank wall).

The eggs were incubated 10 to 15 cm from the surface in groups of five to seven strands placed in plastic baskets or hung by threads from the spray bars. The eggs were examined daily; spent strands (completely hatched) and those with undeveloped embryos or with arrested development were removed. Care was taken to avoid disturbing the embryos, either by sudden mechanical perturbations or light intensity changes, or by extended exposure to light greater than 10–20 lx. Some egg strands in each trial (10–100%) were exposed to an iodine bath (100 ppm for 10 min at 48–96 h intervals) to kill bacteria, protozoans, and microinvertebrates that occurred on and in the egg strand tunic.

Hatchling and production culture

Upon hatching, the squids were reared in hatchery tanks for 4–6 weeks until they were >3–4 cm dorsal mantle length (ML). The incident lighting was increased with an overhead 400-W metal halide light (10–100 lx), but the center of the tank (about 45% of the area) was darker due to the addition of a small circular opaque cover. Water current was also variable due to the plastic egg baskets that hung in the tank. The hatchlings were therefore given a choice of light levels and current patterns. Dead squids and the remains of food organisms were removed by siphoning twice a day.

Juvenile squids were moved from the hatchery tanks to either a production raceway (6.1 m L × 2.4 m W × 0.9 m H, 15,000 l artificial seawater) or a large circular production tank (6.5 m circular by 1.75 m deep, 50,000 l artificial seawater). The filtration systems for the production tanks were similar to those used in the hatchery tanks (Yang *et al.*, 1989; Turk and Lee, 1991). During transfer, squids were caught in plastic beakers (>2 l) or in soft nylon nets, transferred immediately to 30-l ice chests, and transported quickly to the production tanks where the ice chests were submerged and the squids released. The squids were frequently, but not always, anaesthetized with 0.5–1.0% $MgCl_2$ solution to reduce stress during transport (Messenger *et al.*, 1985).

Water quality

Water quality (temperature, salinity, dissolved oxygen, pH, ammonia, nitrite, and nitrate) was monitored separately in each culture tank. Trace elements (Wimex brand) were added biweekly to replace those removed by water filtration and the squids. The pH and salinity were measured three times weekly. Ammonia-nitrogen levels (NH_4-N) were determined using the methods of Solorzano (Strickland and Parsons, 1972); nitrite (NO_2-N) was determined by the Shinn method (as applied to seawater by Bendschneider and Robinson in Strickland and Parsons, 1972); and nitrate (NO_3-N) was determined with prepackaged reagents (Hach Nitro Ver). Ammonia and nitrite were measured weekly, and nitrate was measured monthly or as needed.

Foods and feeding

Food organisms were added to the hatchery tanks soon after the squids hatched, usually within 48 h. Estuarine-collected (Galveston Bay, TX) and hatchery-reared crustaceans and fishes were the primary food organisms during the first 60 d. Larger shrimps and fishes were included as the squids grew. All food organisms were quarantined in filtered seawater for 24 h and were then rinsed with hatchery tank water before being fed to the squids. Food was added frequently, 6–8 times d^{-1} at first (0–60 d), and less frequently (3–5 times d^{-1}) thereafter. Food remains and feces were siphoned out of the tanks daily (1–2 times d^{-1}). During a specific period (days 38–94) of the G_p generation in Trial One, feeding rate was quantified by weighing the food offered to the squids at each feeding and by weighing the amount of uneaten food siphoned from the tank daily. Feeding rates were determined from estimates of squid biomass and the amount of food ingested daily (food offered minus uneaten remains).

Survival and growth

Squid mortalities were counted daily, and a representative sample of freshly dead squids (10–100%) was weighed to the nearest milligram wet body weight (BW) and measured to the nearest millimeter ML. Necropsies were performed when unexplained mortalities occurred. Once the number of squids that died during a trial and the number of squids transferred to a production tank were known, the number of hatchlings with which the experiment began could then be calculated, and survival rates could be estimated. Population growth rates are expressed as instantaneous growth rates (IGR; % BW d^{-1}) over a specific period of the life cycle. The IGR of cephalopods is typically calculated using an exponential function (Forsythe and Van Heukelem, 1987) and the formula is $BW(g) = ae^{b(t_2-t_1)}$, where a is the y-intercept, e the natural logarithm, and b equals the IGR for the time period t_2–t_1.

Daily biomass was estimated from the number of squids surviving each day, and from the recorded live BW of the squid on that day or from the estimated BW per squid for that day based on the IGR.

Behavior

Squid behavior was monitored mainly by observation through the glass windows installed in every hatchery and production tank; video and still photography were used occasionally. Observations were more frequent during initial hatching (1–4 times h^{-1}) and at the onset of reproductive activity (1–2 times h^{-1}) than during the intermediate period of growth and maturation (6–10 times d^{-1}).

Results

Egg transport

Trial One G_p eggs arrived from Japan on 2 September 1987. Ninety-five strands of stage 19–20 (Segawa, 1987) eggs were shipped in four bags at densities of 7.1, 6.7, 4.2, and 3.8 strands per liter of seawater. Transit time, from the beginning of packing to the beginning of unpacking, was 31 h. The temperature on arrival was 21.5°C in all four bags, while the pH and ammonia ranged from 7.50 and 1.55 ppm, respectively, in the most densely packed bag, to 7.70 and 0.91 ppm, respectively, in the least densely packed bag. Although the pH was lower and the ammonia concentrations higher than desired (8.0 and 0.1 ppm, respectively), no correlations between packing density, water quality, or hatching rate were established. Egg transport conditions for Trial Two (24 July 1990) were similar to those of Trial One (initial packing temperature, 20°C).

Egg development and hatching

Field-collected eggs. All field-collected eggs (G_p) were selected at the time of collection by visual inspection of the ova so that the fertility rate of the eggs approached 100% (Table I). At an average of five fertile ova per strand, there were approximately 475 ova shipped for Trial One. Forty-nine percent (235/475) of the ova hatched normally. Trial Two was started with 355 egg strands having an average of 4.1 fertile ova per strand for a total of 1456 embryos. The hatching rate of 37% (542/1466) was lower than that in Trial One. The hatching rates of the G_p eggs were significantly higher ($P < 0.01$) than for the subsequent laboratory-spawned eggs (G_1, G_2, G_3).

Egg strands from both trials were treated with iodine dips (100 ppm); these dips reduced bacterial counts on the surface of the egg tunic by 100-fold, from 10^5 to $<10^3$ colonies cm^{-2}. More concentrated dips (300 ppm) killed all surface bacteria, <10 colonies cm^{-2}. The iodine dips were uniformly effective against all bacteria, since 41 bacterial isolates were exposed to the iodine treatment (100 ppm) and no resistant forms were identified. The egg tunics appeared to deteriorate less when treated than when left untreated, but neither hatching rate nor hatchling survival were correlated with the iodine treatment ($P > 0.01$).

Spawning in the laboratory. At the beginning of the spawning period (148 days post hatching), the Trial One parental generation (G_p) comprised nine females. These females averaged 246 mm ML (range = 215–350 mm ML) and 953 g BW (range = 630–1866 g BW). We cannot be sure that every one of these nine females participated in egg laying, but 1857 G_1 egg strands were laid, an average of 206 egg strands or 1030 total embryos per female (at 5 ova per strand).

In the two subsequent generations (that produced G_2 and G_3 eggs), the spawning period started on days 182 and 230, respectively. The fecundity was 61 egg strands (553 egg strands/9 females) or 305 embryos for each of nine G_1 females, and 659 egg strands (1979 egg strands/3 females) or 3294 embryos for each of three G_2 females. The G_1 and G_2 females that participated in egg laying averaged 260 mm ML (range = 167–312 mm ML) and 1073 g BW (range = 288–1518 g BW) and 214 mm ML (range = 185–230 mm ML) and 682 g BW (range = 578–787 g BW), respectively.

Egg fertility ranged from 49.8% to 73.8% (Table I), and laboratory-spawned egg masses were often flawed mor-

Table I

Egg fertility, hatching rate, and hatchling survival (upon transfer from hatchery to production tanks) for laboratory-cultured Sepioteuthis lessoniana *in Trial One*

Parental generation	Hatchling generation	Strands laid (#)	Percent fertile (%)	Fertile embryos (#)	Hatching rate (%)	Survival in hatching tank (%)
Field collected	G_P	95	≈100.0	≈475	49.0	13
G_P	G_1	1857	73.8	6,850	5.9	40
G_1	G_2	553	73.8	2,040	0.8	29
G_2	G_3	1979	49.8	4,185	1.5	NA[1]

[1] The G_3 hatchlings were reared for only 2 weeks before the trial was terminated.

phologically; thus the hatching rates (0.8–5.6%) were significantly lower than those of the field-collected G_p eggs (49%). Some egg strands did not remain attached to the substrate; others were not sealed at one or both ends, causing the ova to slip out of the tunic sheath; still others were misshapen and contained no ova. All egg strands that appeared normal were incubated for more than 96 h in the production tanks, completing the most critical period of organogenesis. The egg strands were then treated with iodine and transferred to hatchery tanks containing fresh seawater of a temperature and salinity matched closely to that of the production tanks.

Survival and growth

Hatchlings averaged 5.3 mm ML (range 3.5–6.4 mm ML) and 8.2 mg BW (range 4.3–12.0 mg BW) during the three successive generations in Trial One (Fig. 3). The G_1 and G_2 hatchlings survived better in the hatchery tanks than the G_p hatchlings (Table 1; Fig. 4). Most mortalities were the result of premature hatching and starvation, but in a significant number the cause could not be determined. When the juveniles were transferred to the production tanks, survival rates improved to >70% (Fig. 4). During this 150–300 day production phase, the principal causes of death were tank system design (*i.e.*, jumping from tank, inking, and being sucked into the plumbing), 7–18%; cannibalism, 11–17%; starvation associated with cataracts, 2–11%; senescence or unidentified causes, 34–55%; and harvest for scientific experimentation, 22–32%. Cannibalism usually occurred after a squid was already moribund, so most mortalities attributed to cannibalism had another cause initially. More squids could have been used for experiments, but they were kept for broodstock, and eventually died after a period of senescence.

Trial Two consisted of 42 culture days in two hatchery tanks and 333 culture days in three production tanks (two raceways and the large circular production tank). On the night of day 206, the adult squids in the large circular production tank (55,000 l) suddenly became excited and began to ink, so that by morning the entire tank was black with ink. The whole episode was captured on videotape because an increase in irritability of the squids had been noticed and a camera had been installed to monitor the tank at night. Squid densities were the highest yet cultured in our tanks, 5.6 squids m^{-3}, and the nitrates were the highest recorded—94.4 ppm. Therefore, nitrate was believed to be acting as an irritant, initiating the inking behavior that eventually caused the death of all but one squid. In subsequent trials, additional protein skimmers and carbon filtration were added to all production tanks, and nitrate levels have been controlled (<50 ppm) by water exchanges. Deaths due to nitrate-induced inking have thus been avoided.

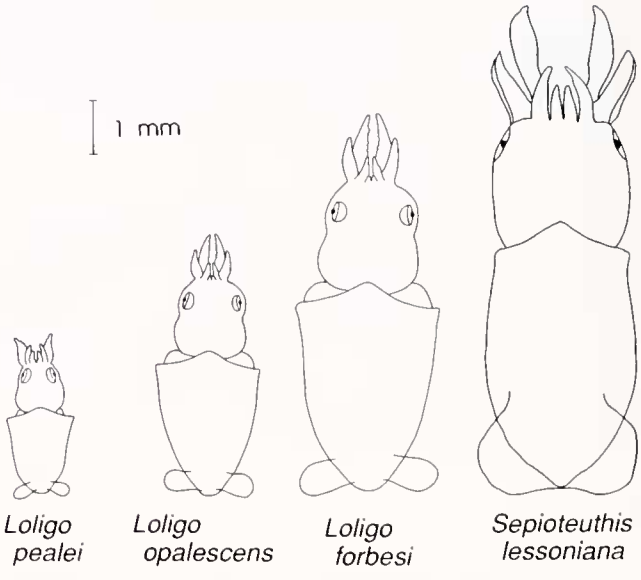

Figure 3. Sizes of various loliginid squids at hatching. Note the large body size and fin of *Sepioteuthis lessoniana*

The instantaneous growth rate (IGR) for the entire life cycle declined with each generation in Trial One (5.3, 4.9, 3.9% BW d^{-1}, respectively), as did the maximum adult size, 2.21, 1.92, and 1.49 kg BW and 360, 328, and 301 mm ML, respectively (Fig. 4). The IGR for the first 100 days of the life cycle was 8.2, 12.0, and 8.5% BW d^{-1}, respectively. One obvious effect of laboratory culture was lengthening of the life cycle. The G_2 generation's life span was 100 d longer than those of the previous two generations. Trial Two produced an IGR of 3.9% BW d^{-1} over the entire life cycle, with a maximum of 8.6% during the first 40 d in the hatchery tank. The maximum adult size was 0.838 kg.

Water quality

High standards for water quality were the goal for all trials, and the control of nitrogen waste products improved with each generation. Ammonia levels were held below the recommended 0.10 NH_4-N ppm except during egg transport and in the G_p generation when both hatchery and production tanks were repeatedly above this level. Although the ammonia concentration in shipping bags (1.5 ppm) was more than an order of magnitude greater than our goal of 0.1 ppm, the hatching rate of the field-collected eggs was significantly better ($P < 0.01$; 49 and 37% in Trials One and Two) than for the laboratory-spawned eggs (G_1, 5.6%; G_2, 0.8%; G_3, 1.5%). The high levels of ammonia present in the Trial One G_p generation were due to the acclimation of the filter bed bacteria to a new temperature regime (21–26°C compared to earlier trials at 11–18°C). In addition, the biomass of squids in

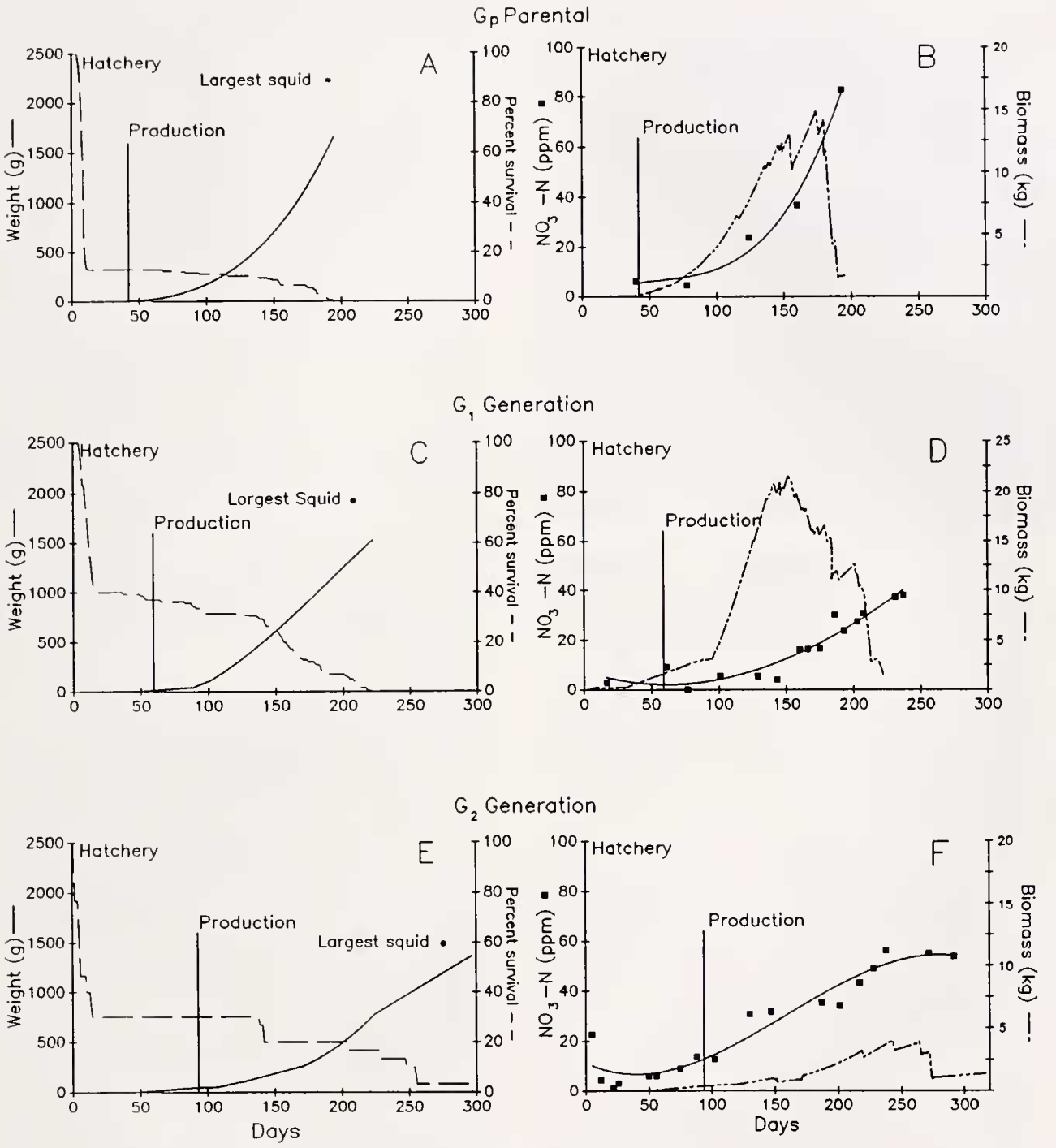

Figure 4. Survival and growth (4A, 4C, 4E) of squids, and water quality and biomass (4B, 4D, and 4F) in Trial One (three consecutive generations). See text.

the production tanks was 10–50 times higher than in previous trials (1–5 kg m^{-3} *versus* 0.10 kg m^{-3}). The increased biomass became a problem also in the production tanks near the end of the G_1 and G_2 generations, but no mortalities could be associated with these increased levels. Nitrite levels (recommended <0.10 ppm) were held below 0.02 NO_2-N ppm in the hatchery tanks and below 0.05 ppm in the production tanks. Nitrate levels NO_3-N were never a problem in the hatchery tanks because of the short duration of the culture period and the low biomass, but the levels in the production tanks eventually exceeded our goal of <50 ppm in the G_p (84 ppm) and G_2 (62 ppm) generations (Fig. 4). Note the similarities in the respective slopes of the biomass and nitrate curves

during the G_p and G_2 generations, confirming the direct effects of squid biomass on nitrate accumulation.

The pH was maintained at 8.0 ± 0.09 in Trial One, and the lowest levels were 7.70, 7.75, and 7.80 for the G_p, G_1 and G_2 generations, respectively. When the pH began to drop, sodium bicarbonate was added to readjust the pH to 8.0; usually several additions over 72 h were required to reestablish a pH of 8.0. The salinity of all tanks in all generations was held above 32 ppt, with the mean being 33.2 ± 0.8. Temperature was steady in all generations, with a mean of 23.2 ± 0.6°C.

Similar patterns of fluctuation in pH (8.1 ± 0.16) and metabolite levels (NH_4-N, 0.02 ± 0.03 ppm; NO_2-N, 0.02 ± 0.03 ppm) were observed in Trial Two, except that nitrate levels increased to 94.4 ppm in the large circular production system immediately before the inking episode described above. Temperature and salinity were held within narrow limits, 23.2 ± 0.9°C and 34.2 ± 1.0 ppt.

Foods and feeding behavior

Live food was added to the hatchery tanks within 48 h of a major hatch (>100 hatchlings) and 6–10 times per day thereafter. Field-collected mysid shrimp (*Mysidopsis almyra*), hatchery-reared penaeid (*Penaeus vannamei*) and palaemonid (*Palaeomonetes* sp.) shrimp larvae and hatchery-reared guppies (*Poecilia reticulata*) were the primary food organisms during the first 60 d of each trial (Fig. 5). Field-collected penaeid shrimps (*Penaeus setiferus* and *Penaeus aztecus*), palaemonid shrimp, and several estuarine fish species were included when available in the appropriate size (<1 cm).

Feeding rates varied from 26 to 33% BW d^{-1} during this 60-d hatchery period. Within the first day of hatching, squids would follow mysids or palaemonid shrimp larvae to the bottom and would even prey successfully upon shrimps and fishes equaling their own size. Within a few weeks, squids could be seen grasping three fishes at once. Food preferences were not clear—squids tried to capture a variety of the prey that were offered (Fig. 5). As with *Loligo*, there seemed to be some squids that were more successful in capturing prey organisms, and the number of unsuccessful attacks decreased gradually during the first few weeks.

After transfer to the production tanks, the squids (>5 g BW) were fed progressively larger shrimp and fishes 3–6 times per day (Fig. 5). The predominant species were the penaeid shrimps (*P. setiferus* and *P. aztecus*) and estuarine fishes (*Cyprinodon variegatus, Poecilia latipinnea*, and *Mugil cephalus*). Other fishes (both field-collected and laboratory-cultured) were added as available. Feeding rates during the entire production phase (days 60–300) varied from 20 to 35% BW d^{-1}, with a mean of 27.8% BW d^{-1}. We noticed that squids several months old would not actively pursue schooling fishes (mullet, *M. cephalus*, and

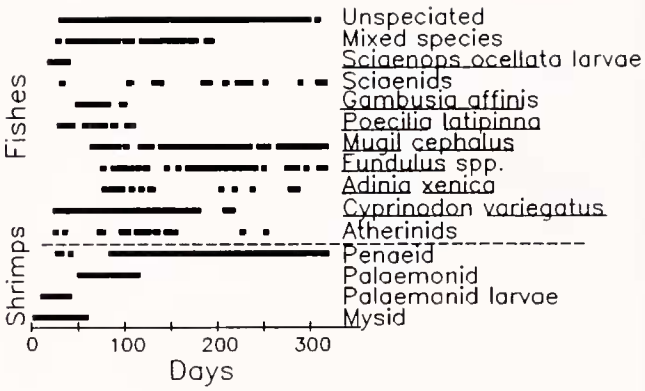

Figure 5. Food timeliness throughout the culture in Trial One. See text.

molly, *P. latipinnea*), except when an individual fish would leave the school. By two months of age, squids had been conditioned to the opening of the tank top, and their response to food was enhanced. The squids showed no preference for either species of shrimp or for any species of fish that was offered during a 56-d period (days 38–94) of the Trial One parental generation (G_p) in which feeding rate was quantified for individual food species.

Effects of light on behavior

Squids were reared in constant low light for most of the life cycle. Young squids generally avoided both the brightest and darkest parts of the tank. Thus, a round piece of black plastic placed over the center of the circular hatchery tank created a shaded central area in contrast to the more brightly lit walls. Squids spent more time in the middle of the tank, resulting in less wall contact and thus less fin damage. However, for the G_1 generation of Trial One, lighting was used to manipulate the behavior of both the squids and their food organisms during the hatchery phase of culture. Four incandescent lamps were placed around the perimeter of the circular hatchery tank, and the light was cycled 15 min on/30 min off throughout the day; this on/off cycle caused the mysid shrimp to move up and down in the water column, enhancing feeding opportunities by the squids. Typical light levels in the hatchery tanks were 85 lx in the bright areas and 5 lx in the shaded portions; in the production tanks the levels were between 10 and 20 lx.

Schooling behavior

The large, robust hatchlings (5 mm ML) were strong swimmers from the outset, and schooling was evident within 2 weeks in every trial. These schools comprised up to 40 individuals, and formed most often when human disturbance in and around the tank was strong. Squids schooled tightly when first transferred into the production

tanks, but within a day or two they acclimated to the tank and dispersed.

Body patterning

A noteworthy feature of the *S. lessoniana* hatchlings was the well-developed ability to produce complex body patterns from the moment of hatching. Figure 2 illustrates one such complex pattern that includes a postural component (Upward V curl of the arms) as well as various chromatic components, most notably the transverse mantle bars. Comparable patterning in other loliginid squid species does not occur before 4 months of age. A complete list of body patterning components does not appear in this publication, but the repertoire of *S. lessoniana* is moderately less diverse than that of *S. sepioidea* in the Caribbean (Moynihan and Rodaniche, 1982) and far more diverse than those of the other species of *Loligo* that we have cultured.

Reproductive behavior

Reproductive behavior occurred throughout the last quarter of the life cycle. Spawning was sometimes accompanied by sudden profuse inking (which in this case was not due to high levels of nitrate). Males became aggressive and engaged in agonistic contests that involved mainly posturing and body pattern displays, but the details have never been documented well. Invariably, when the ink was filtered out of the water within several hours, eggs were found to have been laid in the tank. Mating occurred by two methods: the "head-head" position (seen only twice) and the "male-parallel" position in which the male grasped the female from slightly underneath and inserted the hectocotylus into the mantle cavity of the female (illustrated by Segawa, 1987; Segawa *et al.*, 1993). This latter approach was variable because the male also grasped the female from above, and in one odd observation a male approached a female from underneath by swimming upside down, but no copulation actually occurred. Only temporary mating pairs were seen. On occasion, males were seen to accompany females as they laid eggs; this "mate guarding" has been observed in nature, too (Segawa *et al.*, 1993).

Females required specific substrates on which to lay egg strands: (1) tall, thin artificial plants similar to the Japanese seaweed *Sargassum ringgoldianum* (Segawa, 1987), or (2) arches constructed of polyvinyl chloride pipe, with artificial plants and rocks attached to them. These arches were highly successful—squids laid nearly all their egg strands on these structures.

Giant axons

The giant axon of *S. lessoniana* is very similar in gross morphology to that of other loliginid squids (Fig. 6). The

Figure 6. Giant axon of *Sepioteuthis lessoniana* (560 μm diameter). The dissected axon (≈1 cm distal of cell body) was fixed with a gluteraldehyde and osmium solution, sectioned, and photographed by light microscopy. Bar = 100 μm.

diameters of fresh preparations of giant third-order axons (Fig. 7) were measured under a dissecting microscope after fine dissection and before use in membrane biophysics and axon injury experiments. The measurements were made distal (≈1 cm) to the cell body. In addition to the axon measurements made in the two trials described herein, Figure 7 includes the axon diameters from squids grown in six other culture trials ($N = 137$). The average diameter of the axons from adult *S. lessoniana* (>400 g) was 434 ± 68 μm.

General features of the axon include a large diameter (>400 μm), enabling internal perfusion, internal dialysis, axonal-wire voltage clamp techniques, and video light microscopy of structural changes induced by injury; one giant axon per stellate ganglion (two total per squid); a useful length of giant axon preparation (≈5 cm); a mantle thickness and opacity greater than for other *Loligo*, necessitating improved contrast for quick dissection; cellular injury responses similar to those of *Loligo pealei*; channel types identical to those in *L. pealei*, as verified by similar pharmacological effects of ion channel blockers (tetrodotoxin and 4-amino-pyridine); and electrical impedance similar to those for *L. pealei* (Krause *et al.*, 1992; Krause, 1993). Finally, *S. lessoniana* have been anesthetized repeatedly with $MgCl_2$ during transport from one culture system to another with no mortality. The ability to anesthetize a squid raises new possibilities for both surgical procedures and long-term investigations of chronic injury to the nervous system.

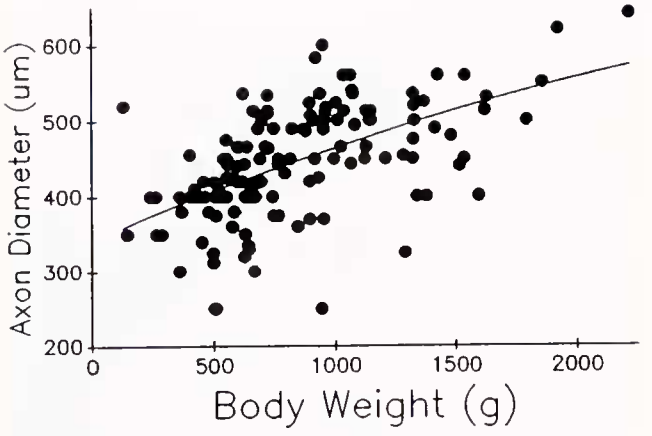

Figure 7. Axon diameters of laboratory-cultured *Sepioteuthis lessoniana* ($N = 137$). Note the large variation relative to body weight.

Discussion

We have successfully cultured *Sepioteuthis lessoniana* through multiple laboratory generations (G_0, G_1, and G_2), and this is the first time that any squid has been cultured through more than one generation (Hanlon *et al.*, 1991). The squid *Loligo opalescens* had been cultured through one generation twice in our facilities, but the G_1 generation was reared for only 10 days (Yang *et al.*, 1986). Other investigators have reared squids through most of one life cycle (Choe, 1966a; LaRoe, 1971; Yang *et al.*, 1980, 1983; Hanlon *et al.*, 1987, 1989; Segawa, 1987) or successfully spawned field-collected adults in the laboratory (Ikeda, 1933; Larcombe and Russell, 1971), but none have cultured squids from egg to egg. Most of the early failures were due to the selection of a species with physiological and behavioral characteristics not amenable to culture, to inadequate tank system design (both engineering and behavioral), to lack of appropriate foods, or to poor water quality.

The large hatchling size (5 mm ML) and high growth rate (>12% BW d^{-1} in the first 100 d) are important biological characteristics for the laboratory culture of *S. lessoniana* because less time is spent in the hatchling and juvenile stages. The fact that *S. lessoniana* is a warm-water squid largely explains its higher growth rate when compared to that of other laboratory-reared squids, which were generally cold-water species (Yang *et al.*, 1980, 1986; Turk *et al.*, 1986; Hanlon *et al.*, 1987, 1989). The behavior of *S. lessoniana* in laboratory tanks was also pivotal to their successful growth and reproduction in captivity. These laboratory-cultured *S. lessoniana* fill a significant gap in the temporal availability of squids for biomedical research.

Behavioral attributes

The outstanding feature of *S. lessoniana* is behavioral adaptability to the confines of the laboratory tanks. In addition to advances in culture technology, the proper selection of a species with behavior amenable to laboratory culture was a key element responsible for the success of this culture program. In nature, *S. lessoniana* occurs inshore, near temperate rock reefs or coral reefs, and this may partially explain its adaptability to tanks (Segawa, 1987; Jackson, 1990). Throughout all life stages, *S. lessoniana* often hovers near vertical structures, and this behavioral attribute—which helps it adapt to the confines of the laboratory—is different from other loliginid squids.

The large fins of *S. lessoniana*—in both hatchlings and adults—allow it to maneuver in and around objects better than other squids. This improved control results in less contact with tank walls, reducing skin damage that has frequently been described for other squids (Summers, 1974; Leibowitz *et al.*, 1977; Hulet *et al.*, 1979). We found, as did LaRoe (1971), that *Sepioteuthis* appears to orient above dark substrates such as it might encounter in nature (*e.g.*, seagrass beds); thus, our production tanks had dark bottoms to calm the squids and keep them away from the walls. Furthermore, possibly as part of the adaptation of this genus to reef structures, the skin appears to be thicker and stronger (although this deserves future examination), resulting in less skin damage when the squids strike the walls or bottom of the tank. As a result, *Sepioteuthis* seems less prone to chronic mantle infections than *Loligo*. The gladius of many *S. lessoniana* adults is broken internally 2–3 cm from the tail due to wall collisions during agonistic contests and courtship behavior. These breaks heal and form scar tissue, but do not become infected secondarily as in *Loligo* (*cf.* Hanlon and Forsythe, 1990). All of these features contribute to the ease with which this species can be caught, handled, and even shipped to potential users at remote locations.

The hatchlings (5 mm ML; Fig. 3) are unique among squids because of their relatively large size and advanced development. Hatchling squids are robust, and the fins are already large and strong, enabling them to capture a wide array of foods. The larger fins and overall size enable them to school within 2 weeks, much earlier than *Loligo* spp. that must grow for 20–60 days before they are large enough to swim independently of currents (Yang *et al.*, 1986; Hanlon *et al.*, 1989). All loliginids studied thus far began schooling when they attained sizes of 4–10 mm ML; *S. lessoniana* is 5 mm ML at hatching.

Lighting affects squid behavior. Hatchling squids stayed near the interface of light and dark areas, and thus light could be manipulated to keep them away from the walls, avoiding skin damage. By maintaining constant low light, we prevented the strong reaction of squids (jetting and hitting the tank walls) to sudden changes in light intensity described by LaRoe (1971) for *S. sepioidea;* yet sexual maturation was not inhibited, as had been reported in some species under constant bright light (Mangold, 1987). Maturation and successful reproduction in all of our cul-

ture trials were not inhibited, but seemed to be accelerated (*i.e.*, 6-month cycle compared with 11- to 13-month cycle in nature; Segawa, 1987).

Mating behavior of *S. lessoniana* was described by Segawa (1987) and Segawa *et al.* (1993), who observed males positioning themselves underneath females (the "male-parallel" position) for 3–4 s while they transferred spermatophores. Our laboratory observations differ somewhat. We observed the "head-head" position, which is common in many loliginid squids (*cf.* Hurley, 1978; Arnold, 1984), and we also have the curious observation of the male approaching the female by swimming upside down while below the female. Segawa (1987) and Segawa *et al.* (1993) noted: "The male remained above the spawning substratum and escorted the female." Our observations corroborate this temporary mate-guarding behavior of males, a tactic that has been observed in other squids (*e.g., Loligo pealei;* Griswold and Prezioso, 1981) and that is common in polygynous animal species. This behavior ensures that fertilized eggs are laid before other males mate with the females (Smith, 1984).

The life cycle was accelerated in the laboratory, and this may have caused the time-limited episodes of reproduction and death. Confining squids at high densities may exaggerate this aspect of their life cycle in comparison to the natural spawning activity of this genus (Tsuchiya, 1981; Segawa, 1987; Segawa *et al.*, 1993).

Culture methods and comparisons

The culture of *S. lessoniana* required significant improvements in five areas of husbandry: (1) design of behaviorally acceptable culture tanks (*i.e.*, size, shape, color, and water flow), (2) water quality management to accommodate high metabolic rate and ink removal, (3) feeding regimen, (4) spawning methodology, and (5) egg handling methodology. The first three of these were particularly important to early hatchling survival and growth, whereas the latter two led to higher fecundity and reduced egg tunic deterioration. These improvements were gradually implemented, beginning with the G_p generation, so that the accrued benefits are evident in Trial One, but especially evident in Trial Two in which high numbers of hatchlings were cultured. The greatest density and biomass of squids were attained in Trial Two, using the large 6.5-m circular production tank. To compare the above improvements with current and previous reports of squid culture, each improvement will be discussed sequentially.

The shape, size, color, and water flow pattern of the culture tanks. These were important factors determining early hatchling survival. Hatchlings tended to swim gently near objects at the edge of shadowed areas and occasionally swam the circumference of the circular hatchery tank. Although tanks of similar volume (1500 l) had been used to rear *Sepioteuthis* spp. (1900 l, LaRoe, 1971; 1200 l, Segawa, 1987), they were rectangular so that hatchlings were concentrated in the corners. For this reason, a circular hatchery tank that is between 1.5 and 2.5 m in diameter and that includes some vertical and horizontal structure (usually the baskets that held unhatched eggs) is recommended. Water depth should be between 75 and 125 cm so that the squids can choose their position. The color and reflectance of the tank walls were important factors in the feeding of hatchlings in earlier squid rearing trials (*e.g.*, LaRoe, 1971; Yang *et al.*, 1983, 1989). Uniformly dark walls are recommended because they reflect less incident light and highlight translucent food organisms. In production tanks, however, a high-contrast speckled wall pattern was used so that the juvenile squids could detect the wall easily, and they very rarely bumped into the walls in these tanks.

Water quality. Water quality was maintained at the highest standards: <0.10 ppm NH_4-N, <0.05 ppm NO_2-N, <50 ppm NO_3-N, and >8.0 pH. The only exceptions to these standards were during egg transport when pH was low (7.5) and ammonia high (1.5 ppm), and near the end of the production cycle when nitrates exceeded 50 ppm. The production of dissolved and suspended organic matter from partially eaten food and squid inking were the major challenges to the maintenance of water quality. Improvements in filtration and water quality have been identified as the reason for improved survival of squids maintained in recirculating seawater tanks (Matsumoto and Shimada, 1980; Yang *et al.*, 1989; Hanlon *et al.*, 1989; Hanlon, 1990). The most effective components of the filtration system were the protein skimmers (or foam fractionators) and activated carbon that removed the black melanin pigment of the ink, as well as other dissolved organic matter; mortality due to heavy inking has been avoided. Declining pH then became the most important water quality parameter, but buffering was provided by the large, submerged oyster shell biofilters and, when necessary, a dose of sodium bicarbonate (Yang *et al.*, 1989). The ever increasing nitrate concentrations were corrected by water exchanges, so that levels were usually less than 50 ppm (G_1 and G_2).

Feeding regimen. Individuals of *S. lessoniana* consumed 20–30% of their body weight in live food organisms each day, placing extraordinary pressure on the available food resources. This high feeding rate equaled estimates from field work (LaRoe, 1971; Segawa, 1990), but was about 2–3 times that for other loliginid squids that have been reared in captivity (*L. opalescens, L. pealei* and *L. forbesi;* Yang *et al.*, 1986; Hanlon *et al.*, 1987, 1989). Food was occasionally limiting in Trial Two. Food is particularly important during the juvenile stage (<100 g) when the squids are growing rapidly (IGR > 10% BW d^{-1}). Food supplies were identified by both LaRoe (1971) and Segawa (1987) as the major factor limiting mass culture.

Table II

Recent biomedical uses for squids

Research topics	Species[1]	References
Membrane biophysics	Sl, Ss, Lp	Fishman, 1993; DiPolo and Beague, 1989; DiPolo *et al.*, 1989; Caputo *et al.*, 1988
Ion channel	Sl, Ss, Lb, Lo, Lp	Gilly *et al.*, in press; DiPolo and Beague, 1993; Correa *et al.*, 1993; Chow, 1991; Fishman and Tewari, 1990; Perozo *et al.*, 1989
Neuron development and injury	Sl, Lb, Lo, Lp, So	Krause *et al.*, in press; Krause *et al.*, 1992; Gilly *et al.*, 1991; Fishman *et al.*, 1990; Tewari and Fishman, 1990; Stein *et al.*, 1989
Cell biology	Sl, Lb, Lp, Ii, So	Lee *et al.*, 1994; Portner *et al.*, 1991; Heming *et al.*, 1990; Mangum, 1990
Muscle biomechanics	Sl, Lb, Lp, Ii, So	Johnsen and Kier, 1993; Kier and Schachat, 1992; Kier, 1991
Chemical reception	Sl, Lb, Lo	Lee, 1994; Gilly and Lucero, 1992; Lucero *et al.*, 1992
Vision and oculomotor physiology	Sl, Lb, Lp, So	Sivak *et al.*, in press; Kito *et al.*, 1992; Langmack and Saibil, 1991; Budelmann, 1990; Fong *et al.*, 1988
Behavior and pharmacology	Lb, Lo	Gilly *et al.*, 1991; Cooper *et al.*, 1990; Hanlon *et al.*, 1990
Melanin synthesis	So	Chedekal *et al.*, 1992; Zeise *et al.*, 1992
Developmental biology	Sl, Lp	Arnold, 1990; Segawa, 1987

[1] Sl, *Sepioteuthis lessoniana*; Ss, *Sepioteuthis sepioidea*; Lb, *Lolliguncula brevis*; Lo, *Loligo opalescens*; Lp, *Loligo pealei*; Ii, *Illex illecebrosus*; So, *Sepia officinalis*.

The development of methods for capturing, transporting, and acclimating large quantities of field-collected food organisms, *i.e.*, mysids, shrimp, and fishes, played a major role in this culture program. In addition to field-collected crustaceans and fishes, laboratory-cultured crustaceans and fishes were fed (<15% of total diet). Moreover, *S. lessoniana* has recently been reared for 50% of its life cycle on dead fish, with no effect on growth or digestive physiology (DiMarco *et al.*, 1993), and the sepioid squid *Sepia officinalis* has been reared for several months on prepared diets (Castro *et al.*, 1993). Research continues to focus on the formulation of economical diets to meet the nutritional requirements of cephalopods (Lee, 1994).

Spawning and egg handling. Eggs shipped overseas should be packed at lower densities to improve water quality during shipment. The recorded values of 7.5 pH and 1.55 ppm ammonia were potential problems (we recommend >8.0 pH and <0.10 ppm ammonia), so that the extra cost of shipping due to added water (<3 egg strands per liter of seawater) would be a good investment. Although hatching rate of the air-shipped, field-collected eggs was significantly greater ($P < 0.01$) than that of the laboratory-spawned eggs (37–49% *versus* <6%), the hatchling survival rate was lower (13% *versus* 29–40%). The lower hatchling survival rate cannot be specifically attributed to poor water quality during transport, but it could have been a contributing factor.

The use of artificial reefs and artificial sea grass in the tanks as spawning substrate proved to be excellent methods for triggering spawning and for collecting the eggs. The daily culling of infertile eggs and repeated exposure to iodine appeared to improve hatching of some egg strands, and these dips significantly lowered the number of bacteria on the surface of the tunics. But many protozoans and microinvertebrate epibionts were found to survive the iodine dips or to recolonize the tunics, so that the deterioration of the tunics could be a result of these organisms and not the bacteria. The hatching rates—especially in subsequent laboratory generations—were low and thus require improvement.

Certain bacteria with antifungal properties are reported to benefit crustacean egg tunics (Gil-Turnes *et al.*, 1989; Gil-Turnes and Fenical, 1992). Moreover, in cephalopod females, the accessory nidamental glands that provide the jelly for egg tunics contain normally growing bacteria (Bloodgood, 1977; Biggs and Epel, 1991). These bacteria have been isolated from the tunics and may serve a similar role to those identified in Crustacea. Iodine dips have not been required for subsequent laboratory-spawned eggs because the incidence of tunic deterioration has decreased, but dips have been continued for wild-collected G_p eggs. Iodine is recommended only when tunic deterioration becomes an overwhelming problem for hatching.

Biomedical uses

The practical result of these improved technologies and the identification of a suitable squid species is that—for the first time—large loliginid squids are available by laboratory culture. This temporal and numerical increase in availability should have a positive effect on several biomedical research fields, particularly axon biophysics, that have been restrained by the limited and seasonal availability of squids (Table II).

The diameter of the axons in this species is highly variable (434 ± 68 μm) and could not be correlated to sex, length, or wet weight (Fig. 7); even some small adults (<350 g BW) had large axons (>350 μm). This confirms quantitatively what many giant axon researchers have reported concerning the weak relationship between squid

size and axon diameter. Neurophysiologists who have used both the cultured squid *S. lessoniana* and the Woods Hole squid *Loligo pealei* confirm the biophysical similarities of the axon preparations (Krause, 1993; Krause *et al.*, in press; Dr. John Russell, Medical College of Pennsylvania, pers. comm.). Thus, supplemental cultured squid stocks could fulfill research needs when squids are not available at Woods Hole.

Prospects for selective breeding. The large variation in axon diameter for differently sized squids (Fig. 7) raises the consideration of selective breeding to produce larger giant axons in smaller squids. The cost savings would be substantial, and recent research indicates that loliginid squids have ample genetic variability to make selective breeding possible. The pelagic squids (*i.e.*, Ommastrephidae) possess low levels of genetic variability (Romero and Amaratunga, 1981; Carvalho *et al.*, 1992) compared to neritic squids (*i.e.*, Myopsida; Carvalho and Loney, 1989; Brierley *et al.*, 1993; Suzuki *et al.*, 1993). Since *S. lessoniana* falls into the latter category, it may be possible to select for specific traits like axon size. No genetic investigations have been conducted with *S. lessoniana*, but this is a topic of current research in our laboratory.

In summary, squid culture is now a reality, and *S. lessoniana* is the species of choice. Laboratory culture is labor-intensive, but *S. lessoniana* has a rapid growth rate that minimizes the life span. The environmental requirements have been described, and *S. lessoniana* has reproduced repeatedly in laboratory tanks. The primary obstacles to large-scale culture are the supply of food and the low hatching rate associated with frequent egg tunic deterioration. Finally, *S. lessoniana* has suitable axons, and its tolerance to anaesthesia and handling raises the possibility that research areas requiring surgical procedures followed by long-term maintenance can be developed successfully.

Acknowledgments

We acknowledge the hospitality and assistance of our Japanese collaborator, Dr. Susumu Segawa, and thank the personnel at Izu Oceanic Park (H. Masuda, Director), the personnel at Tokushima-Ken Fisheries Experimental Station, and John W. Forsythe, all of whom assisted with the collection and transportation of squid egg strands. Many present and past MBI staff members contributed to the success of this project: Dr. William S. Fisher, John W. Forsythe, F. Paul DiMarco, Joanne Hollyfield, William Browning, Vivian Kahla, Lea Bradford-O'Connor, Bobby Day, Mike Harrelson, John Dobias, Douglas Brining, and Ronald Smith. The authors were assisted by Laura Koppe and Kay Kantowski in preparing this manuscript.

We thank Kay Cooper for Figure 6, and John W. Forsythe for Figures 1 and 2. We are grateful for continued support on NIH grants DHHS RR01024 and DHHS RR04226 and the Marine Medicine General Budget Account of the Marine Biomedical Institute.

Literature Cited

Arnold, J. M. 1984. Cephalopods. Pp. 419–454 in *The Mollusca, Vol. 7: Reproduction.* A. S. Tompa, N. H. Verdonk, and J. A. M. van den Diggelaar, eds. Academic Press, New York.

Arnold, J. M. 1990. Evolution and intelligence of the cephalopods. Pp. 3–9 in *Squid as Experimental Animals.* D. L. Gilbert, W. J. Adelman, Jr., and J. M. Arnold, eds. Plenum Press, New York.

Asian Development Bank/Infofish. 1991. *Cephalopods.* AOB/Infofish Global Industry Update, Infofish, Kuala Lumpur, Malaysia. 127 pp.

Biggs, J., and D. Epel. 1991. The egg-capsule sheath of *Loligo opalescens:* structure and association with bacteria. *J. Exp. Zool.* **259:** 263–267.

Bloodgood, R. A. 1977. The squid accessory nidamental gland: ultrastructure and association with bacteria. *Tissue & Cell* **9:** 197–208.

Brierley, A. S., J. P. Thorpe, M. R. Clarke, and H. R. Martins. 1993. A preliminary biochemical genetic investigation of the population structure of *Loligo forbesi* Steenstrup, 1856 from the British Isles and the Azores. Pp. 61–70 in *Recent Advances in Cephalopod Fisheries Biology.* T. Okutani, R. K. O'Dor, and T. Kubodera, eds. Tokai University Press, Tokyo.

Budelmann, B. U. 1990. The statocysts of squid. Pp. 421–439 in *Squid as Experimental Animals.* D. L. Gilbert, W. J. Adelman, Jr., and J. M. Arnold, eds. Plenum Press, New York.

Caputo, C., F. Bezanilla, and R. DiPolo. 1988. Currents related to the sodium-calcium exchange in squid giant axon. *Biochim. Biophys. Acta* **986:** 250–256.

Carvalho, G. R., and K. H. Loney. 1989. Biochemical genetic studies on the Patagonian squid, *Loligo gahi* d'Orbigny. I. Electrophoretic survey of genetic variability. *J. Exp. Mar. Biol. Ecol.* **126:** 231–241.

Carvalho, G. R., A. Thompson, and A. L. Stoner. 1992. Genetic diversity and population differentiation of the shortfin squid *Illex argentinus* in the south-west Atlantic. *J. Exp. Mar. Biol. Ecol.* **158:** 105–121.

Castro, B. G., F. P. DiMarco, R. H. DeRusha, and P. G. Lee. 1993. The effects of surimi and pelleted diets on the laboratory survival, growth, and feeding rate of the cuttlefish *Sepia officinalis. J. Exp. Mar. Biol. Ecol.* **170:** 241–252.

Chedekel, M. R., A. B. Ahene, and L. Zeise. 1992. Melanin standard method: empirical formula 2. *Pigm. Cell Res.* **5:** 240–246.

Choe, S. 1966a. On the eggs, rearing, habits of the fry, and growth of some Cephalopoda. *Bull. Mar. Sci.* **16:** 330–348.

Choe, S. 1966b. On the growth, feeding rates and the efficiency of food conversion for cuttlefishes and squids. *Korean J. Zool.* **9:** 72–80.

Choe, S., and Y. Oshima. 1961. On the embryonal development and growth of the squid *Sepioteuthis lessoniana* Lesson. *Venus* **21:** 462–476.

Chow, R. 1991. Cadium block of squid calcium currents. *J. Gen. Physiol.* **98:** 751–770.

Cooper, K. M., R. T. Hanlon, and B. U. Budelmann. 1990. Physiological color change in squid iridophores. II. Ultrastructural mechanisms in *Lolliguncula brevis. Cell Tissue Res.* **259:** 15–24.

Correa, A. M., C. Caputo, R. Morales, and F. Bezanilla. 1993. Characterization of ionic channels in tropical squid giant axons. *Biophys. J.* **64**(2): A92.

DiMarco, F. P., P. E. Turk, J. M. Scimeca, Jr., W. J. Browning, and P. G. Lee. 1993. Laboratory survival, growth, and digestive gland histology of squids reared on living and non-living fish diets. *Lab. Anim. Sci.* **43:** 226–231.

DiPolo, R., and L. Beaugé. 1989. Asymmetrical properties of the Na-Ca exchanger in voltage clamped, internally dialyzed squid axons under symmetrical ionic conditions. *Acta Physiol. Scand. Suppl.* **1989:** P14.

DiPolo, R., and L. Beague. 1993. Effects of some metal-ATP complexes on Na^+-Ca^{2+} exchange in internally dialysed squid axons. *J. Physiol.* **462:** 71–86.

DiPolo, R., L. Beauge, and H. Rojas. 1989. In dialyzed squid axons Ca^{2+i} activates Ca^{2+o}-Na^{+i} and Na^{+o}-Na^{+i} exchanges in the absence of Ca chelating agents. *Biochim. Biophys. Acta* **978:** 328–332.

Dotsu, K., Y. Shima, and Y. Natsukari. 1981. Ecology and fisheries of the oval squid, *Sepioteuthis lessoniana* Lesson in Goto Islands, Nagasaki Prefecture. Pp. 457–465 in *The Life of Goto Islands.* Biological Society of Nagasaki Prefecture.

Fishman, H. 1993. Assessment of conduction properties and thermal noise in cell membranes by admittance spectroscopy. *Bioelectromagnetics* **Suppl. 1:** 87–100.

Fishman, H. M., and K. P. Tewari. 1990. Single Ca^{2+} channels in patches of axosomes from transected squid axon. *Biophys. J.* **57:** 523a.

Fishman, H. M., K. P. Tewari, and P. G. Stein. 1990. Injury-induced vesiculation and membrane redistribution in squid giant axon. *Biochim. Biophys. Acta* **1023:** 421–435.

Fong, S.-L., P. G. Lee, K. Ozaki, R. Hara, T. Hara, and C. D. B. Bridges. 1988. IRBP-like proteins in the eyes of six cephalopod species—immunochemical relationship to vertebrate interstitial retino-binding protein (IRBP) and cephalopod retinal-binding protein. *Vision Res.* **28:** 563–573.

Forsythe, J. W., and W. F. Van Heukelem. 1987. Growth. Pp. 135–156 in *Cephalopod Life Cycles.* Vol. II. P. R. Boyle, ed. Academic Press, London.

Gilbert, D. L., W. J. Adelman, Jr., and J. M. Arnold, eds. 1990. *Squid as Experimental Animals.* Plenum Press, New York. 516 pp.

Gilly, W. F., and M. T. Lucero. 1992. Behavioral responses to chemical stimulation of the olfactory organ in the squid. *J. Exp. Biol.* **162:** 231–249.

Gilly, W. F., B. Hopkins, and G. O. Mackie. 1991. Development of giant axons and neural control of escape responses in squid embryos and hatchlings. *Biol. Bull.* **180:** 209–220.

Gilly, W. F., M. T. Lucero, M. Perri, and J. Rosenthal. In press. Control of the spatial distribution of sodium channels in the squid giant axon and its cell bodies. In *Cephalopod Neurobiology: International Symposium.* J. Abbott, R. Williamson, and L. Maddock, eds. Oxford University Press, UK.

Gil-Turnes, M. S., and W. Fenical. 1992. Embryos of *Homarus americanus* are protected by epibiotic bacteria. *Biol. Bull.* **182:** 105–108.

Gil-Turnes, M. S., M. E. Hay, and W. Fenical. 1989. Symbiotic marine bacteria chemically defend crustacean embryos from a pathogenic fungus. *Science* **246:** 116–118.

Griswold, C. A., and J. Prezioso. 1981. *In situ* observations on reproductive behavior of the long-finned squid, *Loligo pealei. Fish. Bull.* **78:** 945–947.

Hanlon, R. T. 1990. Maintenance, rearing and culture of teuthoid and sepioid squids. Pp. 35–62 in *Squid as Experimental Animals.* D. L. Gilbert, W. J. Adelman, Jr., and J. M. Arnold, eds. Plenum Press, New York.

Hanlon, R. T., and J. W. Forsythe. 1990. 1: Diseases of Mollusca: Cephalopoda. 1.1. Diseases caused by microorganisms; 1.3. Structural abnormalities and neoplasia. Pp. 23–46 and 203–204 in *Diseases of Marine Animals, Vol. III: Cephalopoda to Urochordata.* O. Kinne, ed. Biologische Anstalt Helgoland, Hamburg.

Hanlon, R. T., P. E. Turk, P. G. Lee, and W. T. Yang. 1987. Laboratory rearing of the squid *Loligo pealei* to the juvenile stage: growth comparisons with fishery data. *Fish. Bull.* **85:** 163–167.

Hanlon, R. T., W. T. Yang, P. E. Turk, P. G. Lee, and R. F. Hixon. 1989. Laboratory culture and estimated life span of the Eastern Atlantic squid, *Loligo forbesi* Steenstrup, 1856 (Mollusca: Cephalopoda). *Aquacult. Fish. Manage.* **20:** 15–34.

Hanlon, R. T., K. M. Cooper, B. U. Budelmann, and T. D. Pappas. 1990. Physiological color change in squid iridophores. I. Behavior, morphology and pharmacology in *Lolliguncula brevis. Cell Tissue. Res.* **259:** 3–14.

Hanlon, R. T., P. E. Turk, and P. G. Lee. 1991. Squid and cuttlefish mariculture: an updated perspective. *J. Ceph. Biol.* **2:** 31–40.

Heming, T. A., C. G. Vanoye, S. E. S. Brown, and A. Bidani. 1990. Cytoplasmic pH recovery in acid-loaded haemocytes of squid, *Sepioteuthis lessoniana. J. Exp. Biol.* **148:** 385–394.

Hulet, W. H., M. R. Villoch, R. F. Hixon, and R. T. Hanlon. 1979. Fin damage in captured and reared squids. *Lab. Anim. Sci.* **29:** 528–533.

Hurley, A. C. 1978. School structure of the squid *Loligo opalescens. Fish. Bull.* **76:** 433–442.

Ikeda, H. 1933. Sex correlated markings in *Sepioteuthis lessoniana* Ferussac. *Venus* **3:** 324–329. (In Japanese with English summary.)

Inoha, M., and M. Sezoko. 1968. The experiment on the culture of Aori-ika (white squid) *Sepioteuthis lessoniana. Ryukyu (Okinawa) Fish. Res. Lab.* **1967 Progress Report:** 139–145.

Jackson, G. D. 1990. Age and growth of the tropical near-shore loliginid squid *Sepioteuthis lessoniana* determined from statolith growth-ring analysis. *Fish. Bull. US* **88:** 113–118.

Japan Sea Farming Fisheries Association (JSFFA). 1985. *Farming Fisheries Seed Production, Procurement and Releasing Statistics for 1983.* JSFFA Publication, Tokyo. 298 pp. (In Japanese.)

Johnsen, S., and W. M. Kier. 1993. Intramuscular crossed connective tissue fibres: skeletal support in the lateral fins of squid and cuttlefish. *J. Zool.* **231:** 311–338.

Kier, W. M. 1991. Squid cross-striated muscle: the evolution of a specialized muscle fiber type. *Bull. Mar. Sci.* **49:** 389–403.

Kier, W. M., and F. H. Schachat. 1992. Biochemical comparison of fast- and slow-contracting squid muscle. *J. Exp. Biol.* **168:** 41–56.

Kito, Y., J. C. Partridge, M. Seidou, K. Narita, T. Hamanaka, M. Michinomae, N. Sekiya, and K. Yoshihara. 1992. The absorbance spectrum and photosensitivity of a new synthetic "visual pigment" based on 4-hydroxyretinal. *Vision Res.* **32:** 3–10.

Krause, T. L. 1993. *Rapid Membrane Repair in Two Transected Giant Axons.* Ph.D. Dissertation, University of Texas, Austin, TX. 193 pp.

Krause, T. L., H. M. Fishman, M. L. Ballinger, and G. D. Bittner. 1992. Seal formation in two transected giant axons suggests two models of sealing. *Soc. Neurosci.* **18:** 407–411.

Krause, T. L., H. M. Fishman, and G. D. Bittner. In press. Extent and mechanisms of sealing in transected giant axons of squid and earthworms. *J. Neurosci.*

Langmack, K., and H. Saibil. 1991. Signal transduction in photoreceptors. *Biochem. Soc. Trans.* **19:** 858–860.

Larcombe, M. F., and B. C. Russell. 1971. Egg laying behaviour of the broad squid *Sepioteuthis bilineata* (*lessoniana*). *N. Z. J. Mar. Freshwater Res.* **5:** 3–11.

LaRoe, E. T. 1971. The culture and maintenance of the loliginid squids *Sepioteuthis sepioidea* and *Doryteuthis plei. Mar. Biol.* **9:** 9–25.

Lee, P. G. 1994. Cephalopod nutrition; fueling the system. *Mar. Behav. Physiol.* **25:** 35–51.

Lee, P. G., W. L. Lee-Jane, J. J. Salazar, and V. Holoubek. 1994. The absence of formation of benzo[α]pyrene/DNA adducts in cuttlefish (*Sepia officinalis,* Mollusca: Cephalopoda). *Environ. Mol. Mutagen.* **23:** 70–73.

Leibowitz, L., T. R. Meyers, R. Elston, and P. Chaney. 1977. Necrotic exfoliative dermatitis of captured squid (*Loligo pealei*). *J. Invertebr. Pathol.* **30:** 369–376.

Lu, C. C., and R. W. Tait. 1983. Taxonomic studies on *Sepioteuthis* Blainville (Cephalopoda: Loliginidae) from the Australian region. *Proc. R. Soc. Victoria* **95:** 181–204.

Lucero, M. T., F. T. Horrigan, and W. F. Gilly. 1992. Electrical responses to chemical stimulation of squid olfactory receptor cells. *J. Exp. Biol.* **162:** 231–249.

Mangold, K. 1987. Reproduction. Pp. 157–200 in *Cephalopod Life Cycles, Vol. II: Comparative Reviews.* P. R. Boyle, ed. Academic Press, New York.

Mangum, C. P. 1990. Gas transport in the blood. Pp. 443–468 in *Squid as Experimental Animals.* D. L. Gilbert, W. J. Adelman, Jr., and J. M. Arnold, eds. Plenum Press, New York.

Matsumoto, G., and J. Shimada. 1980. Further improvement upon maintenance of adult squid (*Doryteuthis bleekeri*) in a small circular and closed-system aquarium tank. *Biol. Bull.* **159:** 319–324.

Messenger, J. B., M. Nixon, and K. P. Ryan. 1985. Magnesium chloride as an anaesthesic for cephalopods. *Comp. Biochem. Physiol.* **82C:** 203–205.

Moynihan, M., and A. F. Rodaniche. 1982. *The Behavior and Natural History of the Caribbean Reef Squid* Sepioteuthis sepioidea. *With a Consideration of Social, Signal and Defensive Patterns for Difficult and Dangerous Environments. Advances in Ethology* **25.** Verlag Paul Parey, Berlin.

Nagata, W. D., and H. Hirata. 1986. Mariculture in Japan: past, present, and future prospectives. *Mini Review Data File Fish. Res.* **4:** 1–38.

Okutani, T. 1980. Biology of Cephalopoda—6. Systematics and life history of the Loliginidae. *Aquabiology* **1980:** 20–25.

Perozo, E., F. Bezanilla, and R. DiPolo. 1989. Modulation of K channels in dialyzed squid axons. ATP-mediated phosphorylation. *J. Gen. Physiol.* **93:** 1195–1218.

Portner, H. O., D. M. Webber, R. G. Boutilier, and R. K. O'Dor. 1991. Acid-base regulation in exercising squid (*Illex illecebrosus, Loligo pealei*). *Am. J. Physiol.* **261:** R239–R246.

Romero, M. C. L., and T. Amaratunga. 1981. Preliminary results of biochemical-genetic population structure study of the squid, *Illex illecebrosus.* Third Annual Meeting Northwest Atlantic Fisheries Organization, *NAFO SCR Doc. 81:IX:103.* Ser. No. N405.

Saso, N. 1979. On the culture of aori-ika, *Sepioteuthis lessoniana.* Pp. 85–86 in *25th All Japan Fisheries Workers Activity Results Reporting Convention Materials.* All Japan Federation of Fisheries Cooperation Association. (In Japanese.)

Sato, H., and T. Tsuzaki. 1984. Seed production of *Sepioteuthis lessoniana* Lesson. *Saibai* (*Sea Farming*) **29:** 20–23. (In Japanese.)

Segawa, S. 1987. Life history of the oval squid, *Sepioteuthis lessoniana,* in Kominato and adjacent waters central Honshu, Japan. *J. Tokyo Univ. Fish.* **74:** 67–105.

Segawa, S. 1990. Food consumption, food conversion and growth rates of the oval squid *Sepioteuthis lessoniana* by laboratory experiments. *Nippon Suisan Gakkasihi* **56:** 217–222.

Segawa, S., T. Izuka, T. Tamashiro, and T. Okutani. 1993. A note on mating and egg deposition by *Sepioteuthis lessoniana* in Ishigaki Island, Okinawa, Southwestern Japan. *Venus* **52:** 101–108.

Sivak, J. G., J. A. West, and M. C. Campbell. In press. Growth and optical development of the ocular lens of the squid (*Sepioteuthis lessoniana*). *Vision Res.*

Smith, R. L. 1984. *Sperm Competition and the Evolution of Animal Mating Systems.* Academic Press, Orlando, FL.

Stein, P. G., H. M. Fishman, and K. P. Tewari. 1989. Axoballs: spontaneous formation of large vesicles after injury of squid axons. *Biophys. J* **55:** 588a.

Strickland, J. D. H., and T. R. Parsons. 1972. A practical handbook of seawater analysis. *Fish. Res. Board Can. Bull.* **167:** 1–310.

Summers, W. C. 1974. Studies on the maintenance of adult squid (*Loligo pealii*). II. Empirical extensions. *Biol. Bull.* **146:** 291–301.

Suzuki, S., A. Kuwahara, and K. Washio. 1983. Study on the fishing conditions and an ecological aspect of squids, *Loligo edulis budo* and *Sepioteuthis lessoniana* in the coastal waters off Kyoto Prefecture. *Bull. Soc. Fish. Oceanogr. Res.* **42:** 21–27.

Suzuki, H., M. Ichikawa, and G. Matsumoto. 1993. Genetic approach for elucidation of squid family. Pp. 531–536 in *Recent Advances in Cephalopod Fisheries Biology.* T. Okutani, R. K. O'Dor, and T. Kubodera, eds. Tokai University Press, Tokyo.

Tewari, K. P., and H. M. Fishman. 1990. Rapid production of axosomes: a novel ion channel preparation from squid giant axon. *Biophys. J.* **57:** 319a.

Tsuchiya, M. 1981. On the spawning of the squid, *Sepioteuthis lessoniana* Lesson at Amitori Bay, Iriomote Island, Okinawa. *Bull. Inst. Oceanic Res. Dev., Tokai Univ. Notes* **3:** 53–75.

Tsuchiya, M. 1982. On the culture of the squid, *Sepioteuthis lessoniana* Lesson (Loliginidae), from hatching to the whole life in Iriomote Island, Okinawa. *Bull. Inst. Oceanic Res. Dev., Tokai Univ. Notes* **4:** 49–70. (In Japanese with English summary.)

Turk, P. E., and P. G. Lee. 1991. Design and economic analyses of airlift *versus* electrical pump driven recirculating aquaculture systems. Pp. 271–283 in *Engineering Aspects of Intensive Aquaculture.* Proceedings from the Aquaculture Symposium at Cornell University, Northeast Regional Aquacultural Engineering Service, Ithaca, NY. *NRAES* **49:** 271–283.

Turk, P. E., R. T. Hanlon, L. A. Bradford, and W. T. Yang. 1986. Aspects of feeding, growth and survival of the European squid *Loligo vulgaris* Lamarck, 1799, reared through the early growth stages. *Vie Milieu* **36:** 9–13.

Yang, W. T., R. T. Hanlon, M. E. Krejci, R. F. Hixon, and W. H. Hulet. 1980. Culture of California market squid from hatching—first rearing of *Loligo* to subadult stage. *Aquabiology* **2**(6): 412–418. (In Japanese with English summary.)

Yang, W. T., R. T. Hanlon, M. E. Krejci, R. F. Hixon, and W. H. Hulet. 1983. Laboratory rearing of *Loligo opalescens,* the market squid of California. *Aquaculture* **31:** 77–88.

Yang, W. T., R. F. Hixon, P. E. Turk, M. E. Krejci, W. H. Hulet, and R. T. Hanlon. 1986. Growth, behavior, and sexual maturation of the market squid, *Loligo opalescens,* cultured through the life cycle. *Fish. Bull.* **84:** 771–798.

Yang, W. T., R. T. Hanlon, P. G. Lee, and P. E. Turk. 1989. Design and function of closed seawater systems for culturing loliginid squids. *Aquacult. Eng.* **8:** 47–65.

Zeise, L., R. B. Addison, and M. R. Chedekel. 1992. Bioanalytical studies of eumelanins. 1. Characterization of melanin the particle. XIVth International Pigment Cell Conference Proceedings, *Pigm. Cell Res. Suppl.* **2:** 48–53.